

Amino Acid Pool Sizes, Turnover, and Kinetics in *Spirodela
polyrhyza* Grown Under Photoautotrophic, Mixotrophic, and
Heterotrophic Conditions

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To Noah
my husband, my partner, my best friend
keeper of my sanity and relentless encourager
who taught me how to see the bright side of life

And

To my Mom
who taught me how to get knocked down
day after day
and never stop getting back up

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Introduction

Primary metabolism in plants is responsible for providing the cell with fixed carbon, energy, reduced cofactors for cellular reactions, and all the building blocks for secondary metabolism and production of biomass. This portion of metabolism, including glycolysis, the citric acid cycle, the pentose phosphate pathway, amino acid metabolism, and fatty acid synthesis are relatively conserved across species. Amino acid metabolism is a unique portion of metabolism as it bridges carbon and nitrogen metabolism and amino acids are important intermediates in many metabolic processes. Alanine interacts with glycolysis and the citric acid cycle via pyruvate. Asparagine and its derivatives link through oxaloacetate. Serine connects through photorespiration and feeds into one-carbon/folate metabolism. Arginine plays a key role in nitrogen storage and in the urea cycle. The aromatic amino acids feed into portions of secondary metabolism, namely phenylpropanoid metabolism, and lignin biosynthesis and tryptophan, specifically, provides a substrate for auxin biosynthesis (**Figure 1**).

One of the most fascinating qualities of central metabolism in the context of plants is that they have the ability to run primary metabolism as both autotrophs and heterotrophs. The most basic examples of this are the differences between shoot (metabolic source) and root (metabolic sink) tissue (Sonnewald and Fernie 2018, Ho 1988) and the shifts in metabolism that occur with diurnal cycles when photosynthesis cannot occur (Geiger and Servaites 1994). These

changes have the potential to reveal how plants manage their resources such as fixed carbon from CO₂ in the air, and nitrogen, minerals, and water from the soil.

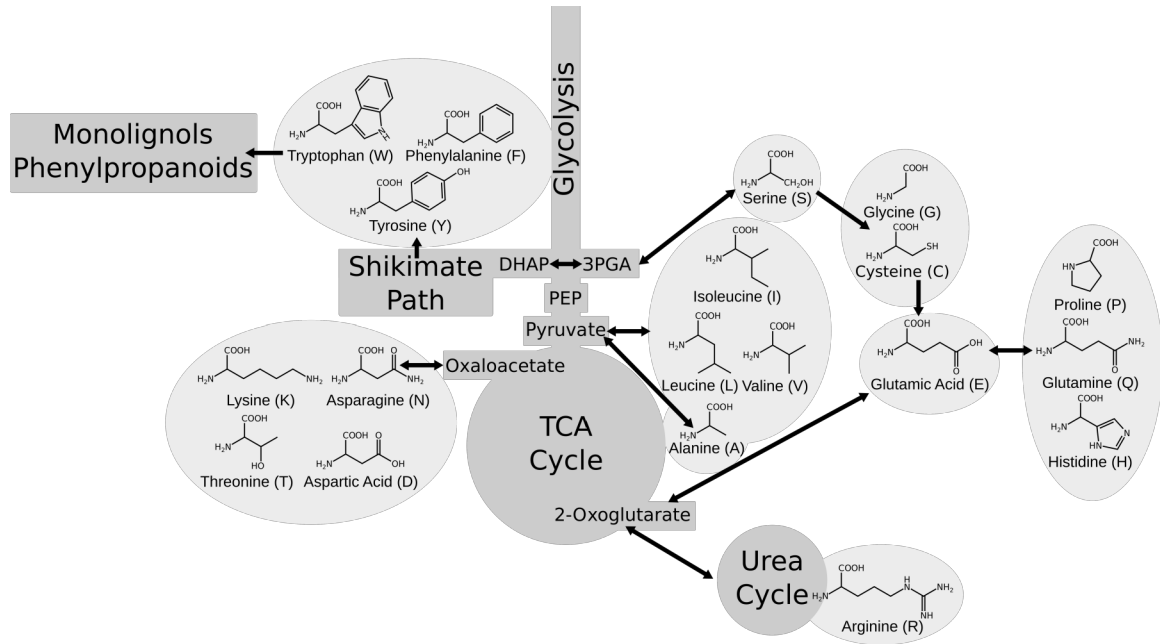


Figure 1. A simple schematic of central metabolism in the context of carbon flow showing the points where the metabolism of various amino acids intersect.

Understanding these plant resource allocation practices can have a potentially large impact on food and plant production (Sonnewald and Fernie 2018) as the end products of central metabolism are the main components of biomass: starch, lipids/fats, and proteins are also the main nutrients people seek in food. The ability to manipulate these core metabolites/nutrients may carry more weight than before as consumer demands and federal regulations of food products are beginning to shift towards healthier macronutrient profiles including improved lipid profiles (Unnevehr and Jagmanaitis 2008).

Light and dark effects on amino acid pools have been well established for a number of years due to many studies carried out in the 1960s through the 1980s (Singh 1998). These studies have assessed amino acid pool sizes in the context of whole plants over the day-night cycle. Each of these studies has shown strong variation in amino acid pool sizes between day and night. The general trend, however, showed that free amino acid pool sizes tended to be lower during the dark night hours (Noguchi and Tomaki 1962, Bauer et al. 1977). This contrasts with studies of chlorophyll synthesis mutants in barley and corn which exhibit accumulation of almost all of the free amino acids (MacLachlan and Zalik 1963, Shortess and Amby 1979). Additional studies in *Zostera marina* root tissue indicated key amino acids, such as glutamate/glutamine and alanine having lower pool sizes in the dark (Pregnall et al. 1987). More recent studies on the transcriptional regulation of key enzymes involved in amino acid biosynthesis support these earlier light/dark amino acid studies (reviewed in Coruzzi 2003).

Metabolic models are curated maps representing how the chemical reactions of metabolism are connected. Recent advances in using annotated genomes to build these networks in a number of plant species including *Arabidopsis thaliana* (Poolman et al. 2009, de Oliveira Dal'Molin et al. 2010a, (Arnold and Nikoloski 2014), corn (de Oliveira Dal'Molin et al. 2010b), and developing embryos (Schwender et al. 2003) have ignited an expansion in the field of metabolic flux and network analysis which seeks to estimate the quantitative flow of matter through metabolic reactions. Many of these advances

have been in the realm of stoichiometric methods, such as flux balance analysis and they have been possible because of the emerging high-quality metabolic models (Sweetlove and Ratcliffe 2011). These methods have been used to elucidate costs associated with amino acid biosynthesis under different nitrogen and metabolic growth conditions (Arnold et al. 2015), resource allocation during seed filling (Schwender et al. 2006), and overall metabolic patterns in central metabolism (Szecowka et al. 2013). However, these studies have had the advantage of having been conducted with species that have well-developed genomes as well as genome-scale metabolic models. In contrast, there are many organisms that do not have these well-developed system models.

The species of interest for this study is the common duckweed (*Spirodela polyrhiza*), a small aquatic angiosperm and a member of the family *Lemnaceae* which contains five genera: *Landolita*, *Lemna*, *Spirodela*, *Wolffia*, and *Wolffiella*. The *Lemnaceae* are monocotyledonous plants that reproduce primarily through asexual budding (Appendroth et al. 2013). They are also among the fastest growing angiosperms and are capable of doubling biomass in 16-24 hours depending on the conditions (Peng et al. 2007). Additionally, they are able to accumulate high starch content of up to 75% of the dry weight (Reid and Bielecki 1970, Xu et al. 2012b) and high-protein content. This ability to accumulate a large percentage of biomass as starch has led to some commercial interest in duckweed species as biofuel feedstock and it has been shown that it is possible to increase this starch content under nutrient stresses (Reid and Bielecki 1970,

Cui and Cheng 2015) and also under certain light regimens (Yin et al. 2015). Duckweed species also grow readily on wastewater. A particular example of growth of three isolates on swine lagoon water (Bergmann et al. 2000, Cheng et al. 2002, Xu et al. 2011) highlights the possibility of mixing duckweed production into existing agricultural pipelines. Additional commercial interests for duckweeds include bioremediation (Oron 1994) and recombinant protein production (Xu et al. 2012a). Because of the utility of duckweed for many uses it is going to be increasingly important to have an understanding of the full extent of metabolism, including metabolic rates/fluxes, static pool sizes, and information on how these respond to different growth and metabolic conditions such as differing light treatments.

In this thesis, I present a metabolic [^{15}N]-labeling study of *S. polyrhiza* grown under three metabolic growth conditions: growth in light cycle on media with supplemental sucrose (mixotrophic condition), growth in light cycle on media without supplemental sucrose (photoautotrophic condition), growth in dark on media with supplemental sucrose (heterotrophic condition). *S. polyrhiza* and other duckweeds have a history of use in stable isotopic labeling studies (Rhodes et al. 1981, Baldi et al. 1991, Rapparini et al. 1999) and can readily incorporate label from liquid growth media. The [^{15}N]-labeling study of *S. polyrhiza* facilitated the ability to measure amino acid pool sizes, turnover numbers, and adjusted pool sizes and to compare between experimental conditions. Here we lay the

groundwork for further in-depth metabolic studies as well as reveal insights into central metabolism in duckweed.

Material and Methods

***S. polyrhiza* material and long-term culture maintenance**

All duckweed cultures used in this work were clonally propagated *Spirodela polyrhiza* [ID number 7498 from the Rutgers Duckweed Stock Cooperative (<http://www.ruduckweed.org/>)] maintained on Schenk and Hildebrandt (SH) media prepared from SH Basal Salt Mixture (Sigma-Aldrich St. Louis, MO) with 1% agar with the pH adjusted to 5.8 using potassium hydroxide. Cultures were kept at 15°C, with a 15 hr light cycle.

Experimental growth conditions

For experiments conducted in the light, actively growing duckweed cultures were maintained on sterile media prepared from SH Basal Salt Mixture (Sigma-Aldrich St. Louis, MO) with the pH adjusted to 5.8 using potassium hydroxide, either with or without sucrose depending on experimental conditions. Cultures were maintained in a controlled growth environment for four to six weeks, under a 16/8 day/night cycle of cool white florescent light at an intensity of $67 \mu\text{mol}/\text{m}^2\cdot\text{s}$ at 22°C. When the cultures reached saturation, the culture was transferred to labeled media as described below.

For experiments conducted in the dark, actively growing duckweed cultures were maintained on sterile SH media prepared from SH Basal Salt Mixture at pH 5.8 (Sigma-Aldrich St. Louis, MO) supplemented with 3.01×10^{-3} mM kinetin as used for *Lemna gibba* (Slovin and Tobin 1982) in a dark box at

room temperature under 2 minutes of red light every 8 hours ($10 \mu\text{mol}/\text{m}^2\cdot\text{s}$) at room temperature. Cultures were only removed from the dark box under green light. When the cultures reached saturated growth, the culture was transferred to labeled media for sampling as described below.

[^{15}N]-labeling experiment media preparation

[^{15}N]-labeled modified SH with a) no added sucrose; b) 10% w/v sucrose; c) 10% w/v sucrose and 3.01×10^{-3} mM kinetin, was prepared according to the specifications in **Table 1**. The pH of the solution was adjusted to 5.8 with potassium hydroxide and autoclaved. The necessary iron components were added by syringe filter sterilization after the media cooled to minimize precipitation of media components.

Table 1. Final concentration for components of the [^{15}N]-labeled growth media.

Media Component	Concentration (mM)
K^{15}NO_3	21.88
$\text{CaCl}_2 \times 2\text{H}_2\text{O}$	1.36
$\text{MgSO}_4 \times 7\text{H}_2\text{O}$	1.62
$^{15}\text{NH}_4^{15}\text{NO}_3$	2.61
KH_2PO_4	2.61
H_3BO_3	8.09×10^{-2}
MnSO_4	6.62×10^{-2}
ZnSO_4	6.19×10^{-2}
Na_2MoO_4	4.86×10^{-4}
$\text{CoCl}_2 \times 6\text{H}_2\text{O}$	4.20×10^{-4}
KI	6.02×10^{-3}
$\text{CuSO}_4 \times 5\text{H}_2\text{O}$	8.01×10^{-2}
FeSO_4 (or NaFe-EDTA)	9.87×10^{-2}
Na_2 (only with FeSO_4)	5.95×10^{-2}
Kinetin(dark experiments only)	3.01×10^{-2}

Duckweed transfer and sampling procedure

Experiments with a light cycle

Duckweed from actively growing cultures +/- sucrose were decanted from the culture under aseptic conditions and washed with sterile distilled deionized water. Duckweed fronds were then sorted into 5 groups and transferred into either five 2 L Pyrex bottles with 300 mL of medium, or five 500 mL Pyrex bottles with 100 mL of medium. Once duckweeds were transferred, initial samples were taken from the extra plant material not needed for the labeling and transferred to pre-weighed 1.5 mL microcentrifuge tubes and snap frozen in liquid nitrogen. Samples were taken at the following time points: 0, 1, 2, 4, 8, 16, 32, 64, and 128 hours. Additional samples were also taken at 15 and 30 min timepoints from cultures grown without added sucrose. Snap frozen samples were stored at -80°C until extraction and LC-MS analysis. Two experiments were carried out for the light without sucrose conditions, which were started at different times in the day. One of these, started later in the day, was used for pool size calculation, and the other, started near the beginning of the light cycle in line with the other light experiment, was used to estimate label enrichment and estimate the kinetic model.

Experiments in the dark

The transfer and sampling procedures take place the same as for light cycle experiments with the following exceptions. Sampling was carried out in the dark

under green light. Samples were taken at the following time points: 0, 15, and 30 min, and 1, 2, 4, 8, 16, 32, 64, and 128 hours.

Microscopy

Duckweed plants were grown in either light or dark conditions for at least two weeks. When cultures reached saturation individual duckweed fronds were removed and thin layers of leaf tissue were scraped off with a clean razor and put onto a microscope slide. Brightfield and fluorescence images were taken at 1000x magnification with Leitz Laborlux D (Stuttgart, Germany) fluorescence microscope. Fluorescence images were acquired with an N2.1 filter to visualize chlorophyll (excitation wavelength range: 515-560 nm (green); emission wavelength: >590 nm (red)). Image J was used to make montages, overlays, and to add scale bars. (Schindelin et al. 2012, Schindelin et al. 2015).

Sample extraction

Frozen sample weights were taken. Samples were kept on dry ice and up to 1.5 mL/mg sample fresh weight cold 70% isopropanol was added to each sample. Samples were then homogenized in a Geno/grinder tissue homogenizer at 1,500 rpm for five minutes. Sample homogenate was then centrifuged at room temperature for at least three minutes and supernatant was decanted into a fresh microcentrifuge tube and stored at -80 °C until mass spectral analysis.

Dry residue analysis

100 or 200 μL of sample extract was added to a pre-weighed 1.5 mL microcentrifuge tube and the samples were then dried under vacuum and the weight of the residue was determined. Samples were reconstituted to a concentration of 2 mg sample extract/mL in 70% isopropanol and stored at -80°C until mass spectral analysis.

LC-MS analysis

LC sample preparation

Extracted samples were removed from storage and centrifuged at room temperature for at least 2 min to settle particulate matter. Samples were then loaded into LC-MS autosampler vials. Fresh plant extracts were diluted 1:10 in 70% isopropanol. Dried extract samples were diluted to a concentration of 1 $\mu\text{g}/\mu\text{L}$ in 70% isopropanol in the light experiment without supplemental sucrose and the dark experiment and to a concentration of 0.9 $\mu\text{g}/\mu\text{L}$ in the light with sucrose experiment. A commercially available [^{15}N]/[^{13}C]-labeled amino acid standard (Sigma-Aldrich St. Louis, MO) was added to dry residue samples for a final concentration of 0.02 mM. All measurements were carried out on five biological replicates except in the light with sucrose experiment, which had only three replicates.

LC-MS analysis

For each sample, 1 μ L was injected onto a SeQuant ZIC-cHILIC column, 3 μ m particle size, 100 x 2.1 mm using an Ultimate 3000 UHPLC system coupled to a Q-Exactive quadrupole-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA) with a heated electrospray ionization source. A 20 min gradient at a flow rate of 0.4 mL/min with mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) with the following gradient: -2-0 min: 90% B, 0 min: 85% B, 18 min: 40% B, 18-20 min: 40% B. MS analysis used the following settings: full scan mode in positive ionization with a scan range of 50-750 m/z , a resolution of 70,000, a target automatic gain control of 1×10^6 , and a minimum fill time of. Data were collected using Thermo *Xcalibur* software version 4.0. Amino acid identity was verified through comparison of retention times and accurate mass to a commercially available mixed amino acid standard. The amino acid isomers of leucine and isoleucine are quantified together because of the inability to separate these with the chromatography system employed.

Data analysis

Exact mass calculation and retention time determination

Exact masses for both labeled and unlabeled amino acids were calculated using the University of Wisconsin - Madison Biological Magnetic Resonance Data Bank exact mass calculator (http://www.bmrb.wisc.edu/metabolomics/mol_mass.php).

Retention times and exact mass measurements of all amino acids were determined using amino acid standard H (Sigma-Aldrich, St. Louis, MO).

MS file conversion and data extraction

Data files were converted from *.RAW* files to *.mzXML* files using the “msconvert” function of *Proteowizard* (Kessner et al. 2008) prior to input into *R*. Data from each amino acid was extracted through use of a pair of scripts developed in the Hegeman lab currently available on GitHub (<https://github.com/orgs/HegemanLab> files “metabolite turnover” and ‘clustering’). Briefly, we utilized the *ProteinTurnover* (Fan et al. 2016) and the *XCMS R* packages (Smith et al. 2006, Tautenhahn et al. 2008, Benton et al. 2010) to extract amino acid extracted ion chromatograms (EICs) for each amino acid isotopomer. These data were then used to generate labeling patterns tracking the decay of the unlabeled isotopomer (M0).

Data modeling

Labeling patterns for M0 output by the clustering script were then used to model the labeling pattern of each amino acid via the ‘nls’ function in *R*. The broom package was used to clean the data for export. The model implemented was a variation of the model by Yuan et al. (2008) for modeling a first-order decay of the unlabeled (M0) isotopomer:

$$y = (1 - c)e^{-kt} + c$$

Where t is the labeling time in hours, k is the turnover constant, and c is a constant that defines the plateau of the labeling curve. Models were generated using time points 0-16 hrs except for the following cases that used 0-32 hrs: lysine in the dark grown experiment and 0-64 hrs: tryptophan in the light grown experiment without supplemental sucrose and alanine, arginine, threonine, and tryptophan in the dark grown experiment. Histidine was omitted from all modeling and valine was omitted from the light without sucrose and dark with sucrose experiments due to low data quality. All models were estimated using five biological replicates except in the light without sucrose experiment, which had only three replicates.

Clustering

K -means clustering was performed using R , time points between 0-32 hrs with k selected to maximize the between-group variation and to minimize the within group variation. The Hartigan-Wong algorithm was used to form clusters based on Euclidean distance. Further clustering was carried out based on the pool sizes adjusted for turnover. The number of clusters used for these analyses were the same as used for the first analysis except in the case of the light with sucrose experiment where four clusters rather than 3 were used.

Estimation of pool size, pool size corrected for turnover, and active pool size

Pool sizes in all experiments were estimated for the initial time point to provide insight into the amount of available amino acid in each condition. Pool sizes were

estimated relative to the labeled internal standard added to dry residue samples.

All numbers for pool size carry the units $\mu\text{mol}/\text{mg}$ sample extract residue.

The k -values from each model was used to estimate a flux value, denoted as pool size adjusted for turnover, for each amino acid as follows:

$$f_{AminoAcid} = k_{AminoAcid} \times P_{AminoAcid}$$

Where $f_{AminoAcid}$ is the flux estimate through an amino acid, $k_{AminoAcid}$ is the turnover number modeled for an amino acid, and $P_{AminoAcid}$ is the initial pool size estimated for an amino acid. All flux dimension numbers or turnover corrected pool sizes carry units of $\mu\text{mol}/\text{mg}$ sample extract residue/hour.

Pool sizes were corrected to approximate an “active pool” as observed in illuminated Arabidopsis rosettes (Szecowka et al. 2013) and maize (Arrivault et al. 2016). The c component in the models were taken as an approximation of the proportion of the pool not active in metabolism and the pool size estimates were corrected as follows:

$$P_{Corrected} = P_{Initial} - (P_{Initial} \times c)$$

Where P_{Active} is the estimated corrected pool size, $P_{Initial}$ is the originally estimated pool size, and c is the modeled plateau for each amino acid.

Pairwise statistical comparisons

Estimates for pool size and turnover adjusted pool size were compared across the three pairs of experimental conditions: light with sucrose vs. light without

sucrose, light with sucrose vs. dark with sucrose, and light without sucrose and dark with sucrose. Comparisons were made via two-tailed student's t -test assuming unequal variance except where a F -test indicated equal sample variances in which case an equal variance test was applied.

Results

[¹⁵N]-labeling of *S. polyrhiza* amino acids in phototrophic, heterotrophic, and mixotrophic growth conditions

In order to confirm the physiological effects of the light conditions duckweed grown for at least two weeks in light or dark conditions were sectioned and imaged under both brightfield and red fluorescence (**Figure 2**).

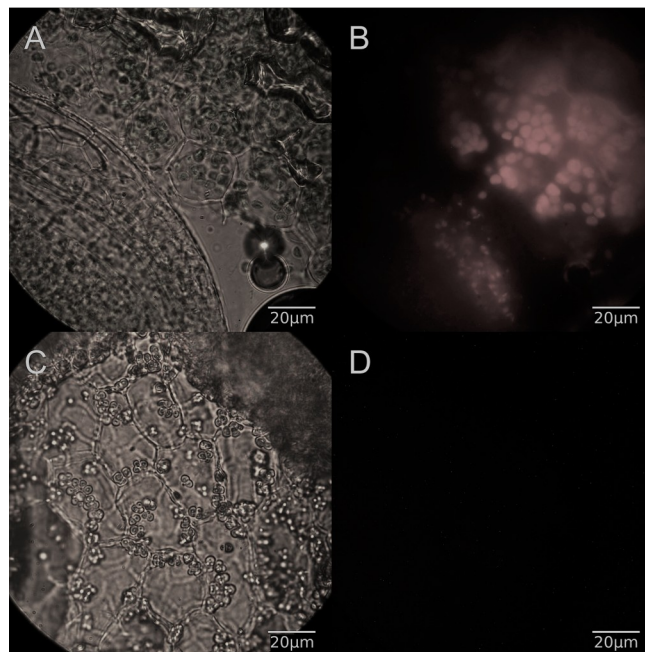


Figure 2. A) Brightfield microscopy of light grown duckweed at 100x, B) Red fluorescence image of light grown duckweed, C) Brightfield image of dark grown duckweed, D) Red fluorescence image of dark grown duckweed.

Light grown samples exhibited normal chlorophyll autofluorescence and as expected dark grown samples had no detectable chlorophyll autofluorescence.


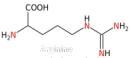








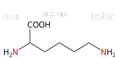







[¹⁵N]-labeling was conducted once the growth conditions were confirmed as phototrophic, heterotrophic, and mixotrophic based on chlorophyll content.

Duckweed fronds that were cultured for at least two weeks were transferred from natural abundance media to [^{15}N]-labeled media and samples were taken at 0, 15, and 30 min and 1, 2, 4, 8, 16, 32, 64, and 128 hrs, except for the light with sucrose treatment, which omitted the 15 and 30 min sample points. Fifteen amino acids were identified by utilizing LC-MS and retention time windows; their isotopomer molecular ion masses are in **Table 2**.

[^{15}N]-Label incorporation models for individual amino acids

Models for the incorporation rate of [^{15}N]-label into each individual amino acid were generated. **Figure 3** contains a visual representation of each model and the values for the parameters of the models are listed in **Table 3**. General labeling patterns can be seen for all amino acids as well as between each experimental condition. Most amino acids exhibit a plateau or leveling-off phase around the 16 hr time point with some amino acids, most notably glutamate, taking longer to reach that plateau. Dark grown samples generally exhibited slower turnover and higher plateau values with several amino acids, including alanine, asparagine, serine, and threonine not appearing to reach fully discernable plateau values within the timeframe measured. Overall, the trends observed by modeling the rate of [^{15}N]-incorporation indicate there are both dramatic and subtle trends that are seen when duckweed is grown in phototrophic, heterotrophic, and mixotrophic conditions.

Table 2. Amino acid retention time ranges for each experiment and estimated exact mass values for each isotopomer

Name	Retention Ranges (Min)	# Nitrogens	M+H	M+1	M+2	M+3	M+4
 Alanine (A)	Light With Sucrose: 4.55-5.8 Light Without Sucrose: 4.8-5.4 Dark with Sucrose: 4.8-5.4	1	90.0549	91.05198	-----	-----	-----
 Arginine (R)	Light With Sucrose: 15.9-17.5 Light Without Sucrose: 14.8-16 Dark with Sucrose: 14-16	4	175.1189	176.11598	177.11302	178.1101	179.1071
 Asparagine (N)	Light With Sucrose: 7.6-8.6 Light Without Sucrose: 7.6-8.6 Dark with Sucrose: 7.6-8.6	2	133.0607	134.057803	135.0548379	-----	-----
 Aspartic Acid (D)	Light With Sucrose: 7.4-8.9 Light Without Sucrose: 10.2-13 Dark with Sucrose: 12-16	1	134.0448	135.0418	-----	-----	-----
 Glutamic Acid (E)	Light With Sucrose: 7.15-8.55 Light Without Sucrose: 7.2-8 Dark with Sucrose: 7.2-8	1	148.0604	149.0575	-----	-----	-----
 Glutamine (Q)	Light With Sucrose: 6.9-8.3 Light Without Sucrose: 7-7.8 Dark with Sucrose: 7-7.8	2	147.0764	148.0735	149.0705	-----	-----
 Glycine (G)	Light With Sucrose: 6.1-7.4 Light Without Sucrose: 6.1-6.7 Dark with Sucrose: 5.5-7	1	76.0393	77.0363	-----	-----	-----
 Histidine (H)	Light With Sucrose: 16.2-17.9 Light Without Sucrose: 15.4-16.8 Dark with Sucrose: 15.4-16.8	3	156.0768	157.0738	158.0708	159.0679	-----
 Isoleucine (I)	Light With Sucrose: 2.6-3.8 Light Without Sucrose: 2.7-3.2 Dark with Sucrose: 2.7-3.2	1	132.1019	133.0989	-----	-----	-----
 Leucine (L)	Light With Sucrose: 2.6-3.8 Light Without Sucrose: 2.7-3.2 Dark with Sucrose: 2.7-3.2	1	132.1019	133.0989	-----	-----	-----
 Lysine (K)	Light With Sucrose: 16.4-18 Light Without Sucrose: 15.4-16.6 Dark with Sucrose: 14.8-16.6	2	147.1128	148.1098	149.1069	-----	-----
 Phenylalanine (F)	Light With Sucrose: 2.65-3.85 Light Without Sucrose: 2.9-3.4 Dark with Sucrose: 2.9-3.4	1	166.0863	167.0833	-----	-----	-----
 Proline (P)	Light With Sucrose: 3.75-5.05 Light Without Sucrose: 4-4.5 Dark with Sucrose: 4-4.5	1	116.0706	117.0676	-----	-----	-----
 Serine (S)	Light With Sucrose: 7.95-9.5 Light Without Sucrose: 8.1-8.8 Dark with Sucrose: 8.1-8.8	1	106.0499	107.0469	-----	-----	-----
 Threonine (T)	Light With Sucrose: 5.9-7.3 Light Without Sucrose: 6.1-6.8 Dark with Sucrose: 6.1-6.8	1	120.0655	121.0626	-----	-----	-----
 Tryptophan (W)	Light With Sucrose: 2.95-4.15 Light Without Sucrose: 3.25-3.55 Dark with Sucrose: 3.25-3.55	2	205.0972	206.0942	207.0912	-----	-----
 Tyrosine (Y)	Light With Sucrose: 4.5-5.75 Light Without Sucrose: 4.8-5.3 Dark with Sucrose: 4.8-5.3	1	182.0812	183.0782	-----	-----	-----
 Valine (V)	Light With Sucrose: 2.95-4.3 Light Without Sucrose: 1.8-2.1 Dark with Sucrose: NA	1	118.0863	119.0833	-----	-----	-----

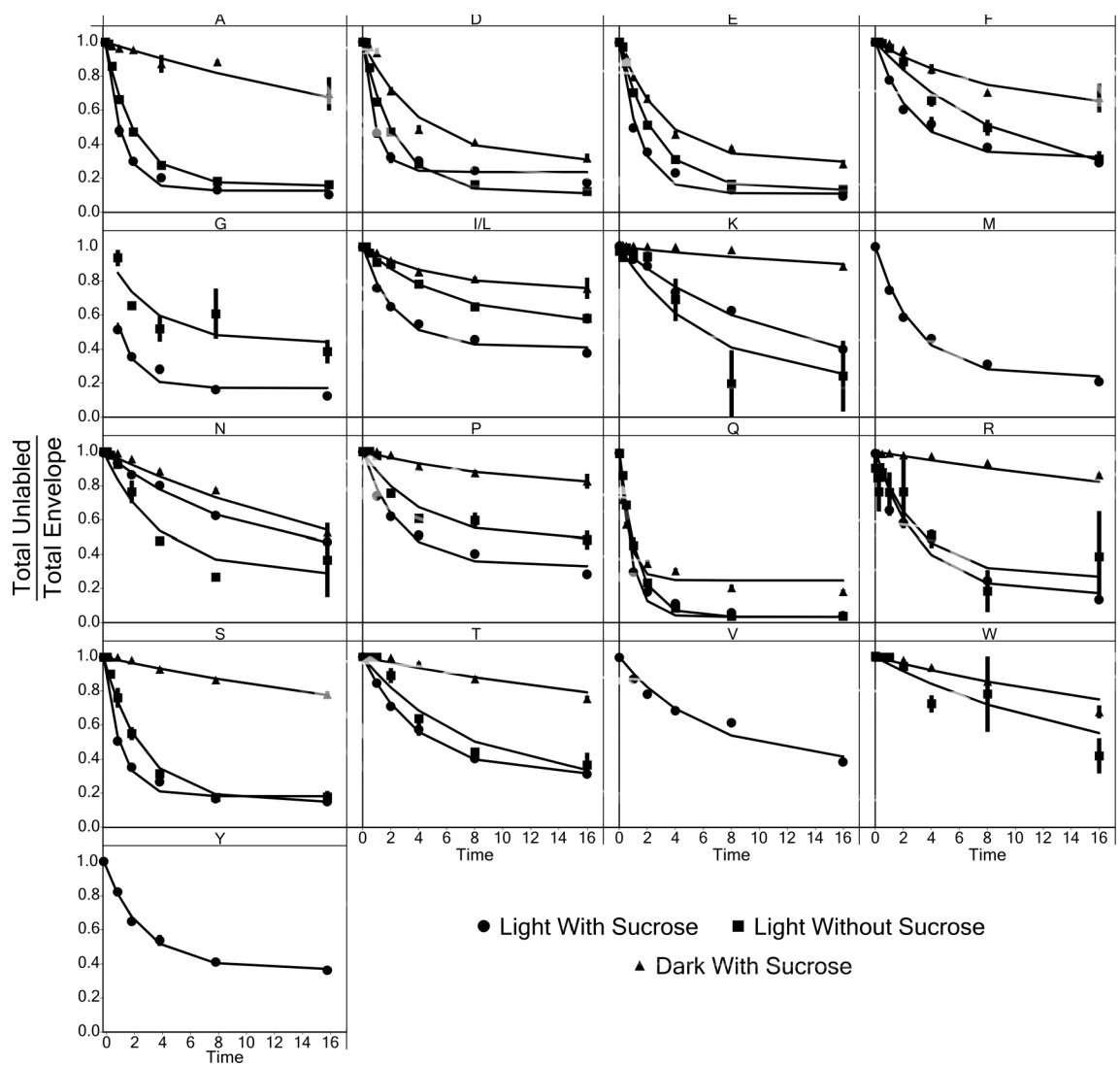


Figure 3. A graph showing the average ratio of the unlabeled isotopomer to the total isotopic envelope for each amino acid in each experiment. The lines represent models generated in R. Error bars represent ± 1 standard error.

Table 3. The modeled values for turnover number (k) and the [^{15}N] labeling plateau value c for amino acids measured in *S. polyrhiza*. All values had a statistical goodness of fit of $p < 0.01$ except where noted in parentheses.

Amino Acid	Experiment	k -value	Standard Error (k)	c -value	Standard Error (c)
Alanine	Light With Sucrose	0.8485	0.0512	0.1281	0.0131
	Light Without Sucrose	0.4688	0.0296	0.1577	0.0176
	Dark With Sucrose	0.0301	0.0062	0.1500	0.0910
Aspartic Acid	Light With Sucrose	1.1581	0.1065	0.2378	0.0147
	Light Without Sucrose	0.4283	0.0303	0.1121	0.0213
	Dark With Sucrose	0.2464	0.0264	0.2967	0.0297
Glutamate	Light With Sucrose	0.7003	0.0471	0.1106	0.0162
	Light Without Sucrose	0.3951	0.0189	0.1314	0.0144
	Dark With Sucrose	0.3247	0.0183	0.2938	0.0145
Phenylalanine	Light With Sucrose	0.376	0.0324	0.323	0.0196
	Light Without Sucrose	0.1073	0.0229	(0.1516)	0.0986
	Dark With Sucrose	0.1204	0.0440	0.5942	0.0768
Glycine	Light With Sucrose	0.7848	0.0527	0.1707	0.0143
	Light Without Sucrose	0.3175	0.1010	0.4370	0.0656
	Dark With Sucrose	--	--	--	--
Isoleucine/ Leucine	Light With Sucrose	0.4379	0.027	0.4099	0.0116
	Light Without Sucrose	0.1638	0.0204	0.5394	0.0261
	Dark With Sucrose	0.1869	0.0502	0.7450	0.0297
Lysine	Light With Sucrose	0.0891	0.0153	0.216	0.079
	Light Without Sucrose	0.1674	0.0862	(0.2000)	0.1859
	Dark With Sucrose	0.0442	0.0216	0.8000	0.0575
Methionine	Light With Sucrose	0.4378	0.0269	0.4081	0.0117
	Light Without Sucrose	--	--	--	--
	Dark With Sucrose	--	--	--	--
Asparagine	Light With Sucrose	0.3542	0.0187	0.2377	0.0138
	Light Without Sucrose	0.2560	0.0756	0.2782	0.0831
	Dark With Sucrose	0.0424	0.0026	0.0721	0.0251
Proline	Light With Sucrose	0.102	0.0132	0.3416	0.0473
	Light Without Sucrose	0.2483	0.0494	0.4815	0.0406
	Dark With Sucrose	0.0926	0.0478	0.7707	0.0686
Glutamine	Light With Sucrose	0.3873	0.0265	0.3279	0.0153
	Light Without Sucrose	0.8170	0.0486	0.0350	0.0169
	Dark With Sucrose	1.5052	0.1090	0.2491	0.0141
Arginine	Light With Sucrose	1.1695	0.0732	0.0349	0.0126
	Light Without Sucrose	0.3247	0.1522	0.2664	0.1252
	Dark With Sucrose	0.0218	0.0043	0.4000	0.0706
Serine	Light With Sucrose	0.3234	0.0405	0.169	0.0368
	Light Without Sucrose	0.3625	0.0320	0.1492	0.0266
	Dark With Sucrose	(0.0325)	0.0179	(0.4505)	0.2440
Threonine	Light With Sucrose	0.8425	0.0441	0.1847	0.0106
	Light Without Sucrose	0.1356	0.0314	0.2516	0.0852
	Dark With Sucrose	0.0376	0.0056	0.5422	0.0265
Tryptophan	Light With Sucrose	0.2487	0.0161	0.3049	0.0174
	Light Without Sucrose	0.0631	0.0271	0.3000	0.1307
	Dark With Sucrose	0.0384	0.0067	0.4602	0.0434
Valine	Light With Sucrose	0.1642	0.021	0.3713	0.0362
	Light Without Sucrose	--	--	--	--
	Dark With Sucrose	--	--	--	--
Tyrosine	Light With Sucrose	0.3599	0.0243	0.3686	0.0146
	Light Without Sucrose	--	--	--	--
	Dark With Sucrose	--	--	--	--

Estimation of amino acid pool size and pool size adjusted for turnover

Pool sizes in $\mu\text{mol}/\text{mg}$ sample extract residue were estimated by comparison to an internal standard (**Table 4**). General patterns were observed through the experiments with the dark with sucrose condition exhibiting the largest pool sizes in almost every case where an amino acid was observed in the experiment (**Figure 4A**). However, the following amino acids are the exception: aspartic acid, glutamate, glycine, asparagine, and glutamine where the light with sucrose treatment exhibited larger pool sizes than other conditions. The light without sucrose treatment exhibited either the smallest or an intermediate pool size in every amino acid. Pool sizes can be explained to be either active or inactive. Active metabolite pools are those participating in metabolic reactions, while inactive pools are not and may be sequestered in a vacuole or otherwise unable to participate in metabolic reactions (Szecowka et al. 2013). Therefore, in addition to total pool sizes a total active pool size was estimated for each amino acid. For our purposes the active pool size was estimated by correcting the estimated pool size with the plateau value (c) of each model. Interestingly, following this correction the light with sucrose condition carried the largest pool size for all amino acids with the exception of lysine and arginine.

The turnover rates (estimated k -values) were multiplied by the initial pool size estimates for each amino acid to give a dimension of flux (μmol compound/unit time). This quantity is not a true flux value as it will encompass all enzymes and reactions involved in nitrogen transfer through each amino acid.

Table 4. A compilation of pool size and turnover adjusted pool sizes for both the active and inactive pools for each experiment. Pool sizes are given in $\mu\text{mol}/\text{mg}$ sample extract residue and turnover adjusted pool sizes are in units of $\mu\text{mol}/\text{mg}$ sample extract residue/hour

Amino Acid	Experiment	Pool Size	Pool Size (Active)	Turnover Adjusted Pool Size	Turnover Adjusted Pool Size (Active)
Alanine	Light With Sucrose	0.0054	0.0026	0.0046	0.0040
	Light Without Sucrose	0.0031	0.0027	0.0015	0.0012
	Dark With Sucrose	0.0375	0.0318	0.0011	0.0010
Aspartic Acid	Light With Sucrose	0.0142	0.0108	0.0164	0.0125
	Light Without Sucrose	0.0057	0.0051	0.0025	0.0022
	Dark With Sucrose	0.0117	0.0082	0.0029	0.0020
Glutamate	Light With Sucrose	0.0279	0.0248	0.0196	0.0174
	Light Without Sucrose	0.0100	0.0087	0.0040	0.0034
	Dark With Sucrose	0.0166	0.0117	0.0054	0.0038
Phenylalanine	Light With Sucrose	0.0015	0.0010	0.0006	0.0004
	Light Without Sucrose	0.0006	0.0005	0.0001	0.0001
	Dark With Sucrose	0.0028	0.0011	0.0003	0.0001
Glycine	Light With Sucrose	0.0004	0.0003	0.0003	0.0002
	Light Without Sucrose	0.0003	0.0002	0.0001	0.0001
	Dark With Sucrose	-----	-----	-----	-----
Isoleucine/ Leucine	Light With Sucrose	0.0006	0.0004	0.0003	0.0002
	Light Without Sucrose	0.0002	0.0001	3.0E-05	1.00E-05
	Dark With Sucrose	0.0014	0.0003	0.0003	0.0001
Lysine	Light With Sucrose	0.0008	0.0007	0.0001	0.0001
	Light Without Sucrose	0.0002	0.0002	3.0E-05	3.00E-05
	Dark With Sucrose	0.0101	0.0020	0.0004	0.0001
Asparagine	Light With Sucrose	0.2282	0.1503	0.0233	0.0153
	Light Without Sucrose	0.0844	0.0609	0.0216	0.0156
	Dark With Sucrose	0.0400	0.0371	0.0017	0.0016
Proline	Light With Sucrose	0.0010	0.0007	0.0004	0.0003
	Light Without Sucrose	0.0005	0.0002	0.0001	0.0001
	Dark With Sucrose	0.0022	0.0005	0.0002	5.0E-05
Glutamine	Light With Sucrose	0.0704	0.0679	0.0823	0.0794
	Light Without Sucrose	0.0310	0.0299	0.0253	0.0244
	Dark With Sucrose	0.0512	0.0384	0.0770	0.0578
Arginine	Light With Sucrose	0.0007	0.0006	0.0002	0.0002
	Light Without Sucrose	0.0004	0.0003	0.0001	0.0001
	Dark With Sucrose	0.0488	0.0293	0.0011	0.0006
Serine	Light With Sucrose	0.0071	0.0058	0.0060	0.0049
	Light Without Sucrose	0.0056	0.0047	0.0020	0.0017
	Dark With Sucrose	0.0159	0.0087	0.0005	0.0003
Threonine	Light With Sucrose	0.0021	0.0015	0.0005	0.0004
	Light Without Sucrose	0.0010	0.0007	0.0001	0.0001
	Dark With Sucrose	0.0032	0.0014	0.0001	5.0E-05
Tryptophan	Light With Sucrose	-----	-----	-----	-----
	Light Without Sucrose	0.0001	0.0001	8.06E-06	5.60E-06
	Dark With Sucrose	0.0014	0.0008	5.0E-05	3.0E-05

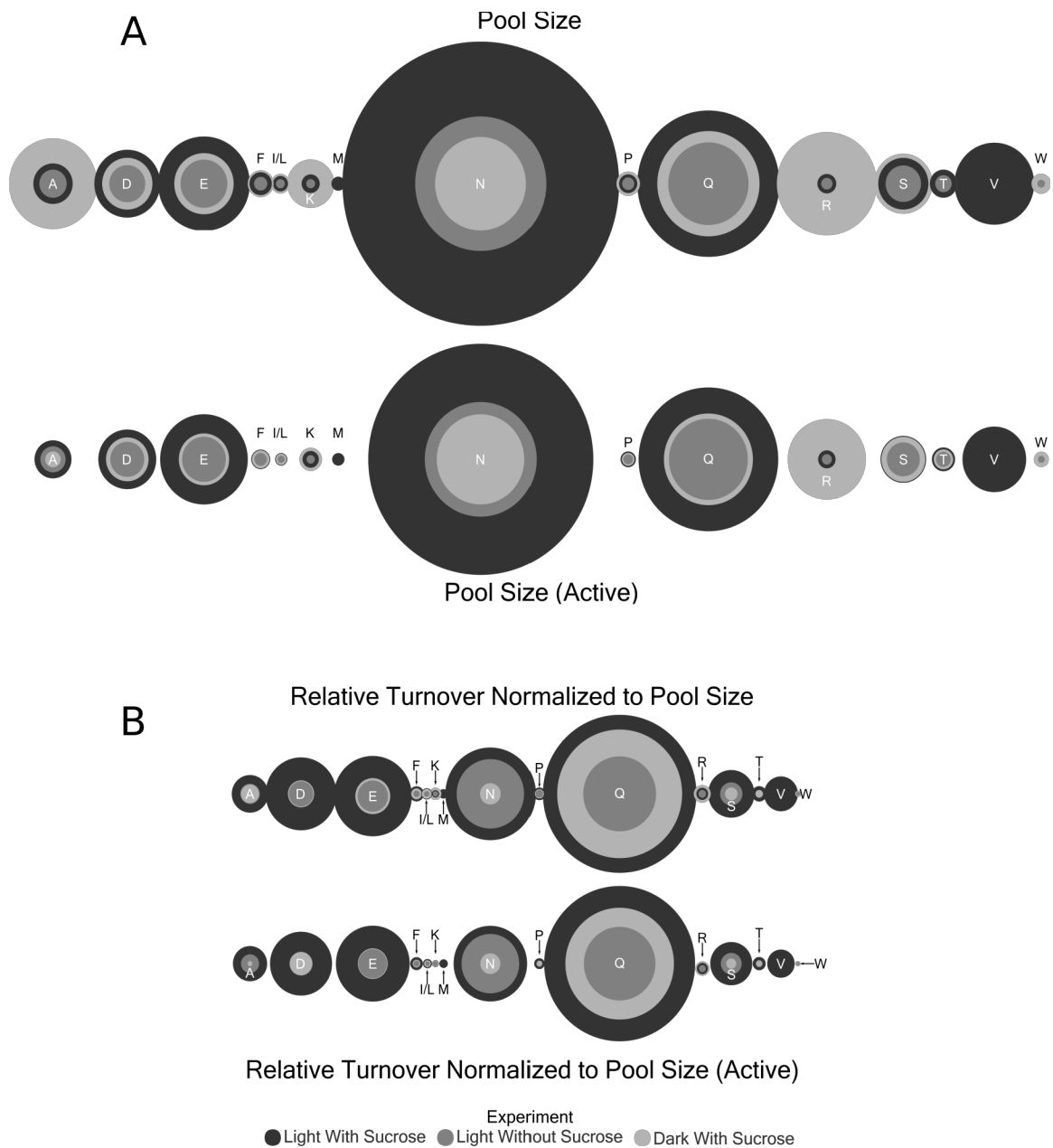


Figure 4. Circle area plots showing a visual representation of relative amino acid pool sizes (μmole) and turnover correct for pool size ($\mu\text{mole/hr}$) in each experiment. Colors indicate the experiment as shown. Circle areas are proportional to the magnitude of the estimate indicated. A) Relative amino acid pool size and active pool size estimates B) Results for turnover normalized to pool size for total and active portions.

The utility of the estimation is to allow direct comparison of turnover rates between amino acids that may have dramatically different pool sizes. Turnover

rates adjusted for pool size in this way in contrast to regular pool size estimates were higher in the light with sucrose experiments in almost all cases (**Table 4** and **Figure 4B**). The exceptions being isoleucine/leucine, lysine, and arginine which both showed larger turnover values in the dark. Similar to the pool size estimation the light without sucrose treatment exhibited either the smallest or an intermediate pool size in every case. However, the light with sucrose and light without sucrose measurements for asparagine were nearly identical.

Turnover rates were also corrected using the active pool size and the results are summarized in **Table 4** and **Figure 4B**. These results largely mirror the uncorrected total pool sizes with a few interesting differences. Lysine showed a compressing of the differences between estimates with the dark grown pool now slightly larger than the light with sucrose pool. In several cases the differences observed in pool sizes between the dark grown and light becomes reduced to the point it is visually indistinguishable. Most notably in aspartic acid, glutamate, proline, and asparagine. In addition, there is a drastic reduction in the alanine turnover corrected for pool size in the dark grown experiment.

Pairwise statistical comparison of pool size and turnover adjusted for pool size

Pairwise statistical comparisons were made between each sample pair (**Table 5**). Many experimental pairs, despite showing appreciable graphical difference did not show statistical significance at least to the $p < 0.05$ level. This may be due to large sample variances in many of the pool size measurements.

Table 5. P-values from pairwise significance tests between each experiment

Pool Size	Alanine	Aspartic Acid	Glutamate	Phenylalanine	Glycine	Isoleucine/ Leucine	Lysine
Light With Sucrose/ Light Without Sucrose		0.022	0.022	0.043		0.021	
Light With Sucrose/ Dark With Sucrose	0.014				----		0.034
Light Without Sucrose/ Dark With Sucrose	0.010			0.020	----	0.041	0.025
	Asparagine	Proline	Glutamine	Arginine	Serine	Threonine	Tryptophan
Light With Sucrose/ Light Without Sucrose	0.019	0.033	0.036				----
Light With Sucrose/ Dark With Sucrose				0.024			----
Light Without Sucrose/ Dark With Sucrose	0.038			0.023			0.016
Pool Size (Active)	Alanine	Aspartic Acid	Glutamate	Phenylalanine	Glycine	Isoleucine/ Leucine	Lysine
Light With Sucrose/ Light Without Sucrose		0.044	0.020				
Light With Sucrose/ Dark With Sucrose	0.012				----		
Light Without Sucrose/ Dark With Sucrose	0.022				----		0.030
	Asparagine	Proline	Glutamine	Arginine	Serine	Threonine	Tryptophan
Light With Sucrose/ Light Without Sucrose	0.025	0.012	0.036				----
Light With Sucrose/ Dark With Sucrose				0.024			----
Light Without Sucrose/ Dark With Sucrose				0.023			0.017
Turnover Corrected Pool Size	Alanine	Aspartic Acid	Glutamate	Phenylalanine	Glycine	Isoleucine/ Leucine	Lysine
Light With Sucrose/ Light Without Sucrose							
Light With Sucrose/ Dark With Sucrose	0.047				----		0.040
Light Without Sucrose/ Dark With Sucrose				0.019	----	0.039	0.030
	Asparagine	Proline	Glutamine	Arginine	Serine	Threonine	Tryptophan
Light With Sucrose/ Light Without Sucrose		0.006			0.012		----
Light With Sucrose/ Dark With Sucrose		0.047					----
Light Without Sucrose/ Dark With Sucrose	0.006		0.048	0.033	0.021		0.019
Turnover Corrected Pool Size (Active)	Alanine	Aspartic Acid	Glutamate	Phenylalanine	Glycine	Isoleucine/ Leucine	Lysine
Light With Sucrose/ Light Without Sucrose					0.049		
Light With Sucrose/ Dark With Sucrose				0.025	----		
Light Without Sucrose/ Dark With Sucrose				0.048	----		
	Asparagine	Proline	Glutamine	Arginine	Serine	Threonine	Tryptophan
Light With Sucrose/ Light Without Sucrose					0.014		----
Light With Sucrose/ Dark With Sucrose							----
Light Without Sucrose/ Dark With Sucrose	0.001			0.036	0.015		0.023

The most significant differences in pool size were present in alanine between the two light experiments and the dark grown experiment as well as asparagine

between the light with sucrose and the dark grown experiment. The results in alanine are preserved in the active pool estimates, while the results for asparagine are not. Additionally, differences in the pool size for proline between the two light experiments emerge when the pool sizes are corrected for a possible active pool. The turnover rates corrected for pool size show the most significant difference in phenylalanine and asparagine between the light without sucrose and the dark grown experiment and in serine between the two light experiments. When an active pool is considered, the differences in phenylalanine become less significant while the differences in asparagine and serine remain significant.

Amino Acids expressed labeling patterns that could be captured by clustering analysis. Cluster composition was not conserved when k -means clustering was applied to turnover rates adjusted for pool sizes

Clustering analysis was performed using a k -means clustering method to assess the similarities that may be present in the label incorporation for amino acids

(Figure 5). The appropriate number of clusters ($k_{clusters}$) was determined empirically by minimizing the within group sum-of-squares variation and maximizing the between group sum-of-squares variation. A $k_{clusters}$ of 3 or 4 was found to be most appropriate depending on the experiment. Broad patterns were seen repeated between treatments using this method. There were distinct patterns in labeling patterns that could be seen between the growth treatments.

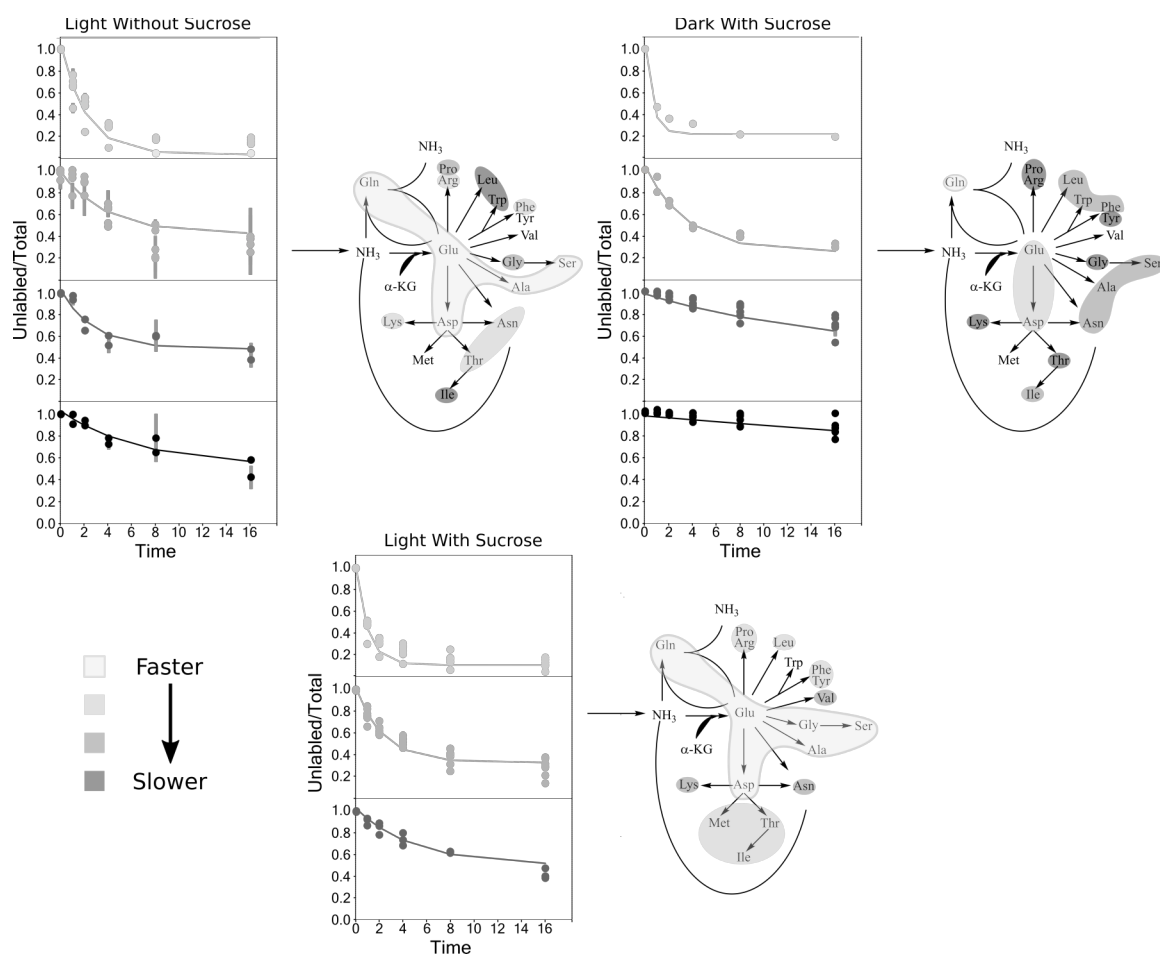


Figure 5. Average labeling patterns following the decay of the unlabeled isotopomer over time for each experiment. The lines are the models generated by *R* and the error bars represent ± 1 SE. Maps adjacent to each set of graphs represent the flow of nitrogen among amino acids. The colored bubbles correspond to the clusters in the above figure highlighting similar isotopic exchange rates for the members of a cluster.

Table 6. A summary of the exponential decay models for each cluster.

Experiment	Cluster	<i>k</i> -value	SE	<i>p</i> (<i>k</i>)	<i>c</i> (constant)	SE	<i>p</i> (<i>c</i>)
Light with Sucrose	1	1.002	0.05	***	0.179	0.01	***
	2	0.437	0.02	***	0.380	0.01	***
	3	0.205	0.03	***	0.530	0.04	***
Light without Sucrose	1	0.472	0.04	***	0.110	0.03	***
	2	0.265	0.07	***	0.400	0.08	***
	3	0.360	0.10	***	0.529	0.06	***
	4	0.149	0.08	---	0.547	0.15	***
Dark with Sucrose	1	1.659	0.12	***	0.278	0.02	***
	2	0.286	0.03	***	0.302	0.03	***
	3	0.061	0.03	**	0.514	0.19	***
	4	0.008	0.08	---	0.000	9.26	---

Glutamine consistently showed the fastest turnover in all experiments, closely followed by glutamate and aspartate. Both light treatments had very similar rapidly turning over clusters consisting of glutamine, glutamate, aspartate, serine, and alanine. However, this grouping is broken up in the dark treatment where glutamine maintained a rapid turnover closely followed by glutamate and aspartate while a number of amino acids involved in carbon and nitrogen central metabolism, including asparagine, serine, and alanine. Each cluster was modeled by a non-linear regression, (**Table 6**).

K-means clustering was also performed using the estimated turnover rates adjusted for pool size for each amino acid individually. Exponential decay models for each amino acid and the clusters generated when considering turnover adjusted for pool size mapped onto a representation of amino acid nitrogen flow are shown in **Table 6** and **Figure 6**, respectively. Several amino acids that seemed to be relatively “core” in the previous analysis (glutamine, aspartate, glutamate, and asparagine) retained their rapid turnover. Some amino acids that fell into apparently rapid turnover segments in the first two analyses, notably glycine and arginine in the light experiments, were shown to have much lower numbers when pool sizes were taken into consideration. This demonstrates that modeling based on the pattern of labeling can be a useful way to categorize metabolites into broad metabolic groups without needing to individually model each amino acid in turn. However, this approach may convolute some details and may also be affected to a certain extent by inactive pools or plateaus in the

labeling pattern and might also not reflect a full picture of the quantity of material moving through a given metabolite.

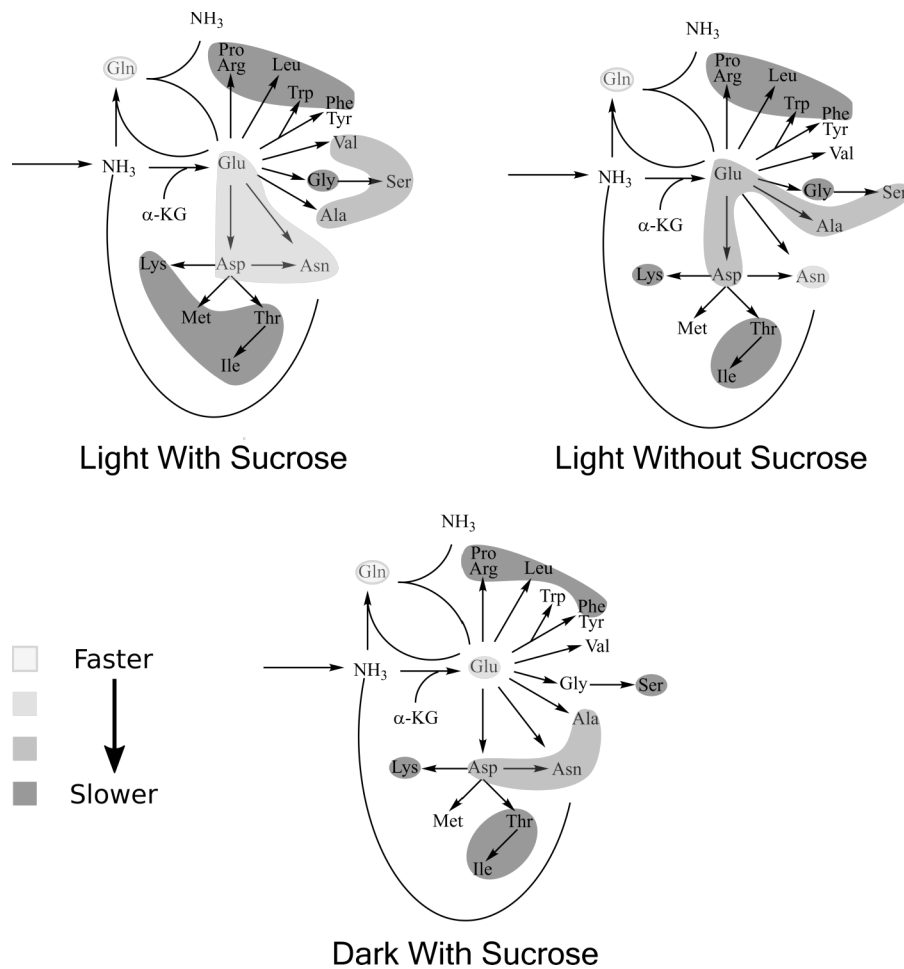


Figure 6. Results of *k*-means clustering for estimates of turnover normalized to active pool size. Colors of each bubble represent the same cluster positions (1-4) and relative exchange rates as in

Discussion

We used stable isotope labeling to model the kinetics of amino acids grown under three different light and/or carbon source conditions 1) A light cycle with 10% w/v sucrose added to the media 2) a light cycle without supplemental sucrose added to the media 3) continuous dark (with 2 minutes red light/8 hours) with 10% w/v sucrose added to the media. Each of these conditions should have the effect of imitating a difference in autotrophic status of the plant from full autotrophic, to mixotrophic, to fully heterotrophic. This difference in plant status can be seen at both macroscopic and microscopic levels (**Figure 2**) as plants grown in light show normal coloration and chlorophyll autofluorescence while those grown in the dark show a bleached color and lack any sign of chlorophyll autofluorescence while still retaining plastid structures.

Labeling kinetics of each individual amino acid also shows interesting patterns. After analyzing and inspecting the data we chose to model the decay of the unlabeled isotopomer following Yuan et al. (2008) with an added term to account for the visible plateau reached by almost all of the amino acids by about 16 hours. Each of these models, with few exceptions, has a very high goodness of fit level ($p < 0.001$) despite somewhat large standard errors in some cases.

Though as noted in a similar analysis carried out by Szekowka et al. (2013) this may be due to an averaging effect across compartments, which could not be resolved in our current analysis. As described above models were generated using the time 0-16 hour points for almost all amino acids. However, for certain

amino acids, most notably many in the dark grown experiment the apparent plateau was not fully realized until closer to the 64 hr time point, if at all.

This failure to reach a plateau can be seen in **Figure 7** for glutamine. Both light experiments have models and labeling patterns that follow each other closely. However, the dark grown experiment begins to deviate from the model after the 16-hour time point. This pattern continues for several amino acids in the dark grown condition, including: aspartate, glutamate, phenylalanine, isoleucine/leucine, lysine, proline, and serine. There are several possibilities that could account for this deviation from the initial models seen in the dark grown samples. The simplest is that, rather than a plateau/inactive pool. It would be more appropriate to model a two-phase labeling consisting of an initial (fast) labeling, as we first measured followed by a slower second phase of labeling as 1) the larger-than-estimated active pool is completely washed out or 2) certain sub-pools/compartments with lower turnover take up label much more slowly. In addition, some irregularities can be observed in the labeling patterns, again in **Figure 7**, and as discussed later regarding metabolic clusters, where glutamine appears to label at a faster rate than glutamate, which seems novel as glutamate is the first entry point for nitrogen during uptake. It is uncertain what causes this labeling pattern, but it is possible that glutamine, which possesses multiple nitrogen atoms, may exhibit two different turnover coefficients, causing one nitrogen atom, most likely in the side chain, to label much more slowly than the alpha nitrogen.

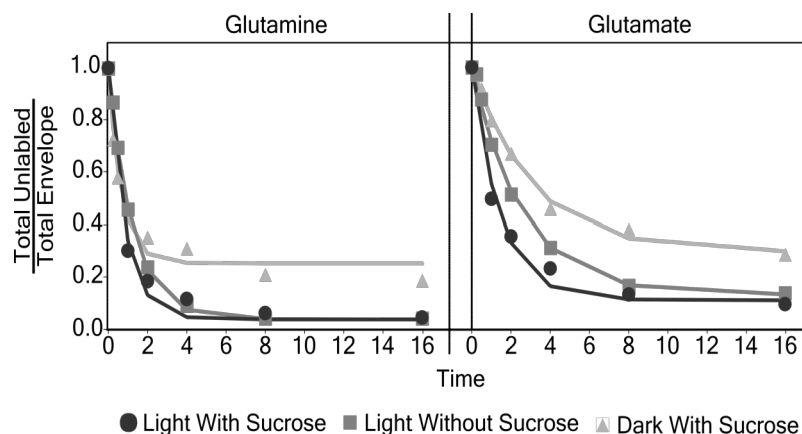


Figure 7. Individual models for glutamate and glutamine for each experiment.

We also used comparison of the time zero samples to a labeled standard to achieve estimates of pool size for most of the amino acids measured. No similar estimates have been carried out in any duckweed species to date to our knowledge making this a unique and useful data set. Several clear patterns emerged in these pool size estimates including the dark experiment showing larger pool sizes in alanine, phenylalanine, isoleucine/leucine, lysine, arginine, serine, threonine, and tryptophan. This result is notable, as many of these amino acids have been shown to accumulate in chlorophyll-deficient mutants during recovery of a normal pigment phenotype (Maclachlan and Zalick 1963, Shortess and Amby 1979). That we see the same patterns between dark grown and light grown with sucrose as was previously seen with some chlorophyll mutants indicates that the differences we are observing likely do come from a difference in autotrophic vs. heterotrophic status of the cell.

In addition to estimating total pool size, we also estimated an active pool size based on the observed plateaus in each labeling pattern. These active pools were defined as the total pool size minus a part of the pool size proportional to the modeled plateau. When this estimation was taken into account many of the amino acids displaying larger pool sizes in the dark no longer displayed larger pool sizes with respect to the light with sucrose treatment, with the exception of lysine, arginine, and serine. This result shows larger inactive pools, overall, in the dark grown experiments. This is significant particularly in asparagine, which is known to be a particularly prevalent nitrogen storage molecule (Lea et al. 2007), and one that is sensitive to the carbon status of the plant as well as arginine, which is generally a primary nitrogen storage molecule in the roots (Winter et al. 2015). As arginine pool sizes seem largest in the dark this could be an indication that metabolism in dark grown duckweed is functioning more like sink tissues such as roots.

Turnover rates were normalized to pool size by multiplying the rate by pool size estimates for each amino acid. By making this adjustment we are able to estimate a quantity with the dimensions of a flux parameter (moles/unit time) that can help better inform the results that we observed in the labeling patterns. While the turnover rates for many amino acids were noticeably slower in the dark, we can see that relatively larger pool sizes of these samples result in more moderate differences between treatments when considering the pool size normalized turnover rate values. This is consistent with the idea that those plants have a

much more robust nutrient supply having carbon available through both photosynthetic carbon fixation and through supplemental sucrose in the media. The notable exceptions to this are arginine and lysine which both show higher pool size adjusted turnover values in the dark. These pool size adjusted turnover rates were also calculated for the estimated active pools and yielded similar results. The one exception is the large decrease in pool size normalized turnover rate when estimated with the active pool size in alanine.

One of the most biologically interesting results is that of serine and the high level of statistical significance found for the difference between pool size adjusted turnover rate in the light experiments with and without sucrose and also between the light without sucrose and dark grown experiments. This is an even more notable result because of a recent interest in serine metabolism. The main source of serine biosynthesis in most conditions is the glycolate pathway (Ros et al. 2014, Douce et al. 2001). This pathway is associated with photorespiration and is thus not expected to be a contributor to serine turnover in conditions where photorespiration is low or nonexistent, such as in a root or an etiolated plant as in the dark grown condition potentially leading to lower turnover overall.

Before attempting individual modeling of amino acids we employed clustering analysis to identify broad patterns in amino acid labeling without the need to calculate individual models. This met with some success. However, when investigating a metabolic system, the most important measure is going to be the pathway flux. While we have not estimated a true flux through each amino acid

for reasons outlined above pool size adjusted turnover rates give us a measurement with the dimensions of flux. When similar clustering analysis is carried out on these normalized rate values general patterns are still observed. However, many clusters now contain only one metabolite giving far less meaningful results. Even with this many of the patterns that are seen in the turnover adjusted for pool size, especially areas of relatively rapid turnover can still be seen. Though, there are some notable changes such as asparagine showing considerably higher turnover than was estimated in the light with sucrose condition through labeling pattern alone. Thus, while useful, due to the fact that they cannot fully capture fluxes and other more quantitative information a clustering-type analysis may be more useful when attempting to investigate metabolic dynamics where full identification and quantification of the involved metabolites is not possible, such as with portions of secondary or specialized metabolism.

Conclusion

In this study stable isotope labeling was used to investigate labeling patterns, pool sizes, and turnover in *S. polyrhiza* under three growth conditions corresponding, roughly to full photoautotrophic growth, mixotrophic growth, and full heterotrophic growth and demonstrated growth of *S. polyrhiza* in the dark for the first time as well as providing the first amino acid pool size estimates for *S. polyrhiza*. These data established the clear labeling plateau and likely presence of metabolically inactive or less active pools in experiments with a light cycle. They also showed that correcting for active pool sizes could, in some cases, have a large effect on comparisons between experiments. We showed that pool sizes are not sufficient to make full metabolic comparisons and that adding a flux dimension, whether via full metabolic flux analysis or the use of turnover number corrected for pool size, can highlight differences that would be otherwise missed in simply considering pool size or turnover number alone.

Labeling patterns in each condition highlighted some interesting known (asparagine pool sizes in the dark) and emerging (serine turnover adjusted pool sizes) trends, particularly, the results regarding serine support the function of photorespiratory pathways as the most efficient/highest turnover mechanism of serine metabolism as we see pool accumulation in conjunction with low turnover in heterotrophic growth conditions. Labeling patterns were also captured by *k*-means clustering analysis. When pool sizes and turnover numbers were considered it became clear that many differences apparent when using those

metrics could be masked when only considering the labeling pattern. However, the agreement that does exist between labeling pattern-derived clusters and turnover normalized for pool size indicate that application of clustering techniques based on labeling pattern could be useful for situations where many compounds are unknown, such as investigation of secondary metabolism.

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